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Association of nitrogen-fixing, plant-growth-promoting rhizobacteria (PGPR) with kallar grass and rice

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Abstract

Leptochloa fusca (L.) Kunth (kallar grass) has previously been found to exhibit high rates of nitrogen fixation. A series of experiments to determine the level of biological nitrogen fixation using ^{15}N isotopic dilution were carried out in nutrient solution and saline soil. These studies indicated an agronomically significant amount of nitrogen being fixed in soil. Kallar grass has a similar growth habitat to rice. Therefore similar studies were carried out with rice after isolating various diazotrophs from the roots which were also screened for their ability to produce auxin (IAA). Five such strains namely *Azospirillum lipoferum* N-4, *Azospirillum brasilense* Wb-3, *Azoarcus* K-1, *Pseudomonas* 96-51, *Zoogloea* Ky-1 were selected for inoculating two rice varieties i.e. NIAB-6 and BAS-370 under aseptic laboratory conditions. The nitrogen fixed was quantified using the ^{15}N isotopic dilution method. Variety BAS-370 had nearly 70% nitrogen derived from atmosphere (Ndfa) when inoculated with *Azospirillum* N-4. Similar studies with the mixed inoculum using ^{15}N fertilizer in the micro plots indicated that nearly 29% of plant nitrogen was derived from the atmosphere.

Introduction

Nitrogen fixation associated with roots of grasses has been recognized as an important component of the nitrogen cycle in a range of ecosystems including several extreme environments (Chalk, 1991). Salinity represents an extreme environment and is characterized by low organic matter and very low nitrogen contents in the soil. However, under these conditions certain plants especially kallar grass (*Leptochloa fusca*) have been found to grow well (Malik et al., 1986). Since the advent of acetylene reduction methodology (ARA), many plants have been shown to harbour diazotrophs in and around their roots (Boddey et al., 1996; Malik et al., 1991). Though certain limitations exist in the application of ARA methodology as reported by Van Berkum (1980) and Witty (1979), its usefulness in screening plants and microorganisms for presence of nitrogenase activity is beyond doubt. Among the plants growing in saline environment kallar grass has been extensively studied with regard to associative nitrogen fixation.

Kallar grass, a highly salt-tolerant plant species, has been recommended as a primary colonizer of salt-affected soils and grows luxuriantly without addition of nitrogenous fertilizers under waterlogged conditions (Sandhu and Malik, 1975). This observation led to the investigation of its rhizosphere for the presence of root-associated nitrogen fixation. Nitrogenase activity associated with the roots in this grass was first reported by Malik et al. (1980, 1982) and a number of diazotrophic bacteria were isolated and characterized from various root fractions (Bilal and Malik, 1987; Bilal et al., 1990; Zafar et al., 1986, 1987). The growth habitat of kallar grass is similar to that of lowland rice. Moreover, rice is generally recommended to be grown on the saline soils where kallar grass had been cultivated. Thus basic information and knowledge obtained from diazotroph-kallar grass association could be directly applied to rice which also grows under similar waterlogged conditions.

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Auxins are growth regulators which are essential for plant growth. Some bacteria are also reported to produce these growth hormones affecting plant growth (Bric et al., 1991; Lifshitz et al., 1987; Tien et al., 1979; Zimmer et al., 1988). The bacteria are known to change the root morphology and increase their biomass, thus enabling them to exploit more of the soil volume and take up soil nutrients. Thus, such bacteria can complement the beneficial effects of nitrogen fixation. Studies have therefore been conducted on the beneficial effects of these bacteria on rice productivity.

Nitrogenase activity and nitrogen fixing bacteria associated with kallar grass

Kallar grass is a perennial grass that has shown high salt-tolerance and can be cultivated with brackish water. Its high biomass yield led us to study its rhizosphere for possible inputs due to biological nitrogen fixation. Our results showed that excised roots of kallar grass exhibited high nitrogenase activity as estimated by ARA (Malik et al., 1982). High acetylene-reducing activities associated with soil and roots (unwashed, washed and surface sterilized) of kallar grass were found (Zafar et al., 1986). Enumeration of diazotrophic bacteria present in the rhizosphere was also carried out (Table 1) using four different culture media (Bilal, 1988). In these studies rhizosphere was separated into various fractions like (1) soil away from roots (NRS), (2) soil adhering to roots (RS), (3) surface of roots (rhizoplane, RP) and (4) interior of roots (histoplane, HP). Nutrient agar medium was used for the enumeration of the total heterotrophic population. Microaerophilic conditions were provided using 0.7% soft agar plating and semisolid media were prepared by using 0.2% agar. Enumerations were based on spread plate technique for aerobes, soft agar overlay for micro aerobic bacteria and most probable number (Cochran, 1950) of ARA positive vials for diazotrophs.

The combined carbon medium (CCM) of Rennie (1981) invariably gave the highest counts of N_2-fixers in all fractions, followed by the malate medium (NFM). Populations differed significantly in rhizosphere soil (RS) and rhizoplane (RP) fractions on CCM medium. The most probable number (MPN) values based on ARA were lower than those obtained by the dilution plate count. The distribution of bacteria was variable in these different fractions. Highest counts were always observed in the rhizoplane fraction on all four media tested (Table 1 and 2).Various diazotrophic bacteria

Table 1. Enumeration of diazotrophic bacterial populations in various rhizosphere fractions of kallar grass using different media

Medium*	Fraction**	Log no. of bacteria/g dry root or soil			
		MPN***	CFU****		
		(ARA-based)	Aerobic	Micro-aerobic	Mean
CCM	NRS	7.00	6.03	8.77	6.81 ab
	RS	6.82	8.23	8.17	7.61 a
	RP	9.61	9.0	8.88	9.44 c
	HP	4.17	6.07	5.95	5.33 a
NFM	NRS	5.05	6.95	7.16	6.32 b
	RS	6.60	7.55	7.88	7.32 bc
	RP	7.77	8.51	9.00	8.47 c
	HP	1.00	5.34	5.30	3.88 a
NFDM	NRS	3.10	8.69	9.14	5.25 a
	RS	5.39	7.88	6.47	6.45 a
	RP	7.47	6.60	8.04	7.51 a
	HP	0.00	5.68	5.57	4.07 a
SSM	NRS	3.00	4.58	7.60	3.80 a
	RS	6.60	7.93	7.92	7.13 bc
	RP	7.92	8.98	9.38	8.76 c
	HP	0.00	5.43	5.64	4.10 a

*= CCM, Rennie, 1981; NFM, Döbereiner et al., 1976; NFDM, Cannon et al., 1974; SSM, Reinhold et al., 1986.
**NRS=soil away from roots; RS=rhizosphere soil; RP=surface of roots; HP=interior of roots.
***=ARA-based most probable number.
****=CFU, colony forming units.

were isolated and characterized (Bilal et al., 1990; Malik et al., 1991). These belong to the genera of *Azospirillum, Azoarcus, Enterobacter, Klebsiella* and *Zoogloea*. It was found that azospirillia were more numerous in the root interior whereas there was preponderance of the genera belonging to Enterobacteriaceae on the root surface. Survival and colonization of the inoculated bacteria in the root rhizosphere was also studied using immunofluorescent techniques (Malik and Bilal, 1989). Some of the common genera of agronomic significance are described below.

Table 2. N_2 fixers in various fractions of kallar grass, estimated by ARA-based MPN method

Medium**	% N_2 fixers*			
	NRS	RS	RP	HP
CCM	1.5	0.014	100	1.36
NFM	0.07	0.080	19	0.00
NFDM	0.01	0.001	4	0.00
SSM	0.01	0.009	10	0.00

*Percent of the total bacterial population; NRS=soil away from roots; RS=rhizosphere soil; RP=surface of roots; HP=interior of roots.
**=CCM, Rennie, 1981; NFM, Döbereiner et al., 1976; NFDM, Cannon et al., 1974; SSM, Reinhold et al., 1986.

Azospirillum

The availability of selective media for isolating diazotrophs belonging to the genus *Azospirillum* and the ease of its detection by its characteristic sub-surface white pellicle in semi-solid agar medium has helped in isolations from the rhizosphere and root surface (Baldani et al., 1986; Döbereiner et al., 1976; Hegazi et al., 1979; Ladha et al., 1987; Sundaram et al., 1988). Several isolates were obtained on semi-solid nitrogen-free media (NFM, CCM) from roots of kallar grass (Bilal and Malik, 1987; Bilal et al., 1990; Malik et al., 1991; Zafar et al., 1987). These isolates formed a fine sub-surface white pellicle in nitrogen-free malate medium within 24 hours, which gradually moved to the surface. The growth in NFM was always accompanied by alkali production and high rates of nitrogenase activity (more than 100 nmol C_2H_4/hour/culture vial). Phase-contrast microscopic examination of wet mounts of the actively growing cultures showed helicle cells resembling *Azospirillum*. The cells were actively motile and exhibited typical spinning motility. Morphologically, *Azospirillum* can be differentiated from all the other bacteria because of its helicle shape from which it derives its name. A high percentage of the isolates accumulated poly-β-hydroxy butyrate (PHB) which was also observed in *Azospirillum* strains by other workers (Reinhold et al., 1986). The majority of the isolates formed light pink colonies when grown on nutrient agar medium, but the pigment was more characteristic and dark pink when nutrient broth-grown cells were pelleted by centrifugation.

None of the isolates used glucose, sucrose and mannitol as carbon sources but grew on lactate, malate and fructose. The biochemical characteristics of the isolates showed that they closely resembled *Azospirillum brasilense* which differs from *Azospirillum lipoferum* in its biotin requirement and ability to utilize glucose as a carbon source (Tarrand et al., 1978). The isolates were compared with various *Azospirillum* species by using one dimensional SDS-PAGE of total bacterial proteins. Comparison with *A. lipoferum*, *A. brasilense* and *A. amazonense* showed that all isolates closely resembled *A. brasilense* strain CdJA (Reinhold et al., 1987).

In a study carried out by Reinhold et al. (1986), nitrogen fixing bacteria were found to form root-zone specific associations with kallar grass, with different populations colonizing the surface and the interior of roots. Two *Azospirillum* species were dominant on the rhizoplane and one of these organisms has been described as a new salt-tolerant species, *Azospirillum halopraferens* (Reinhold et al., 1987a).

Azoarcus

In most of the attempts to isolate diazotrophs from kallar grass, gram negative motile rods were isolated which did not belong to any of the known diazotrophic genera on the basis of the commonly used morphological and physiological tests (Bilal and Malik, 1987; Bilal et al., 1990; Malik et al., 1991; Reinhold et al., 1986). These bacteria have a strictly aerobic type of metabolism, fix nitrogen micro aerobically, and grow well on salts of organic acids but not on carbohydrates. As a result of a polyphasic study carried out by Reinhold-Hurek et al. (1993), a new genus *Azoarcus* was generated to accomodate these isolates from roots of kallar grass collected from Pakistan. One isolate (K-1) from kallar grass was found to belong to this genus by using 16S rRNA oligonucleotide probes (Hassan et al., 1996). Indirect evidence for the colonization of the root interior by these organisms was confirmed by fluorescent antibodies (Reinhold et al., 1987) and immunogold electron microscopy (Hurek et al., 1991). Based on the results of a polyphasic study in which morphological, nutritional and biochemical features and several molecular biological techniques were employed, a new genus *Azoarcus* was proposed with two species *A. communis* and *A. indigenous* (Reinhold-Hurek et al., 1993).

Zoogloea

This nitrogen fixing organism was isolated from the histoplane fraction of kallar grass on CCM medium (Bilal and Malik, 1987). The organism designated as Ky-1 is not one of those commonly occurring on the kallar grass histoplane. Identification of this organism as *Zoogloea* (family Pseudomonadaceae) was primarily based on the presence of a characteristic extracellular capsule or zoogloeal matrix, and also because of its peculiar growth in shaken broth culture, where it aggregates to form macroscopic star-like flocs similar to those of *Zoogloea ramigera* (Krieg and Holt, 1984). The extra-cellular slime produced by Ky-1 is water-insoluble as the culture does not show any turbidity during growth and floc formation. Under the microscope the flocs showed finger-like dendritic out growths, each originating from a cluster of cells. The cells are embedded in a matrix and are static, but

some exhibit rhythmic movements along the margins, whereas free cells in the suspension are actively motile.

Klebsiella, Beijerinckia *and other diazotrophs*

Three diazotrophic bacterial strains were isolated on CCM and named as NIAB-1, C-2 and Iso-2 (Zafar et al., 1987). These bacteria were characterized on the basis of physiological tests and the determination of mole % G+C values of the DNAs. Isolates NIAB-1, C-2 and Iso-2 have mol % G+C contents of 57, 64 and 53, respectively, which were lower than that of the genus *Azospirillum* (68–70%). The characteristics of three isolates were compared with some known nitrogen fixers *Azotobacter, Azomonas, Beijerinckia* and *Klebsiella*. NIAB-1 shared maximum characteristics with the genus *Klebsiella.* Because of the differences within *Klebsiella pneumoniae* in physiological behavior, protein pattern and other molecular characteristics (e.g. 60–70% DNA homology, presence of plasmids), it was proposed that NIAB-1 is a new species of *Klebsiella* (Qureshi et al., 1988) and Iso-2 was identified as *Beijerinckia* .

Quantification of nitrogen fixation in kallar grass by ^{15}N isotope dilution

Quantification of biologically fixed nitrogen in legumes and in grasses has been the most important factor in determining the overall benefit of this process to the cropping system. Nitrogen balance studies have been used most widely as an indication of the extent of nitrogen fixation (Day et al., 1975; Rennie and Larson, 1979). However, the use of $^{15}N_2$ has given values of direct incorporation of fixed N into plants (Eskew et al., 1981; Rennie et al., 1983). This technique can only be used for a short period, during which plants are grown under an enclosed atmosphere and cannot be used in the field. These limitations have made this technique inappropriate for routine use. Techniques based on ^{15}N dilution are however, more versatile and can be adapted to various experimental conditions (Boddey et al., 1996; Roger and Ladha, 1992). ^{15}N isotope dilution techniques have earlier been used for quantifying associative N_2-fixation in wheat (Lethbridge and Davidson, 1983; Rennie et al., 1983), maize (Rennie, 1980), rice (Shrestha and Ladha, 1996; Ventura and Watanabe, 1983), sugarcane (Boddey et al., 1996; Ruschel et al., 1975) and other grasses (Boddey et al., 1983; Boddey and Victoria, 1986; Malik and Zafar, 1985; Malik et al., 1987, 1988).

Several experiments to quantify the amount of nitrogen fixed (Table 3) were carried out both under aseptic conditions and in the field, using the ^{15}N isotopic dilution technique (Malik and Zafar, 1985; Malik et al., 1987, 1988). It has been shown that under sterile conditions 60-80% of the N in aerial parts of kallar grass was derived from fixation by the inoculated bacteria (Malik et al., 1987). In these experiments no additional carbon source was added indicating that root exudation was able to sustain the proliferation of the inoculated bacteria. In the case of a field experiment, the indigenous nitrogen-fixing ability of soil was inhibited by applying a high dose of ^{15}N fertilizer which was taken as the reference treatment. The estimation based on 'A' values (Fried and Broeshart, 1975) indicated that an amount of 32 kg N/ha was derived from fixation resulting in% Ndfa of 26 (Malik et al., 1988).

All the studies reported on kallar grass have indicated the presence of nitrogenase activity associated with its roots and substantial uptake of biologically fixed nitrogen by the plant as demonstrated by ^{15}N dilution technique. Studies on the survival, colonization and attachment of the bacteria to the kallar grass roots have also been reported (Bilal et al., 1993; Malik and Bilal 1989) which indicated that the bacteria colonized and proliferated on the root surface and were also present inside the root hair cells.The bacteria were abundant in the mucigel and around the points of emergence of lateral roots.

N_2-fixation in association with rice

In addition to the kallar grass, diazotroph isolations from rice roots and its rhizosphere were made (Malik et al., 1993). All these isolates were characterized and were found to belong to the genera of *Azospirillum, Azotobacter, Flavobacterium, Pseudomonas, Xanthomonas,* and *Zoogloea*. Five bacterial strains isolated earlier from roots of kallar grass and rice were selected on the basis of their nitrogenase activity and ability to produce IAA for studying their effect on plant growth both individually and as mixed inoculum in pot and field experiments (Table 4). Among the selected strains *A. lipoferum* (N-4) had maximum nitrogenase activity as indicated by ARA values. *Pseudomonas* 96-51 did not show any nitrogenase activity but had maximum IAA production (35 μg/mL). Rice seedlings of variety NIAB-6 and BAS-370 were grown in pots

Table 3. Quantification of N_2-fixation by mixed inoculum of 3 diazotrophic strains in association with roots of kallar grass based on ^{15}N isotopic dilution

Treatment	Control	%Ndfa	Reference	Remark
Lab experiment under sterile conditions	Heat killed E. coli	65–80%	Malik et al., 1987	Plants grown in vermiculite in tubes
Pot experiment	Uninoculated fumigated	Roots 32% shoot 6%	Malik et al., 1987	Saline sodic soil
Field experiment	60 kg N/ha	26%	Malik et al., 1988	Saline sodic soil

Table 4. Characterization of bacterial strains included in the mixed inoculum

Bacterial strain	ARA (nmole C_2H_4/h/mg protein)	IAA production (μg/mL)
Azospirillum lipoferum N-4	686	6.3
Azospirillum brasilense Wb-3	215	16.1
Azoarcus K-1	290	10.5
Pseudomonas 96-51	0	35.7
Zoogloea Ky-1	544	5.1

containing vermiculite and N-free Hoagland nutrient solution. ^{15}N labelled ammonium sulphate of 10 atom % excess (34 mg/pot) was added as a tracer to quantify nitrogen fixation. The plants were harvested after six weeks and plant samples were analyzed for ^{15}N excess on a double inlet Mass Spectrometer (MAT GD 150).

Among the two rice varieties tested, the beneficial effect of bacterial inoculation was more prominent in variety BAS-370 as compared to NIAB-6 (Figure 1). In association with the rice variety BAS-370, *Azospirillum* strain N-4 fixed considerably higher nitrogen as compared to other strains, contributing about 66% of the total N in plants. For rice variety NIAB-6, *Azoarcus* strain K-1 proved to be more efficient, and maximum plant biomass was also recorded with the same strain K-1. Two bacterial strains *Azoarcus* K-1 and *Zoogloea* Ky-1 used in the present study were isolated from kallar grass growing in saline/waterlogged soils of Pakistan (Bilal and Malik, 1987).

Another experiment was performed in cemented micro plots of 1.5 m $\times$ 1.5 m size. Mixed inoculum comprising the five strains listed in Table 4 was used. The inoculum was applied to rice by dipping the roots of one-month old seedlings for 30 minutes before transplanting. Rice variety NIAB- 6 was used. The field experiment was carried out in a randomized complete block design with four replicates for each

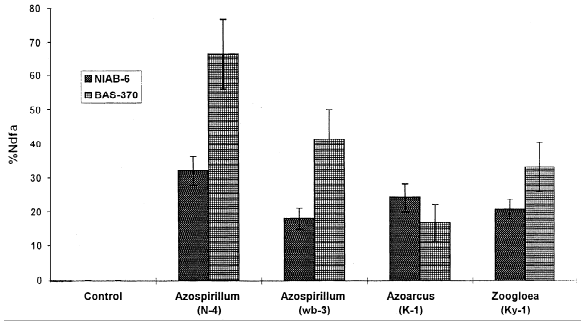

Figure 1. Quantification of N_2 fixation (%Ndfa) by inoculated bacteria in rice.

treatment. Inoculated treatments received 5 atom % ^{15}N fertilizer in the form of ammonium sulfate at 30 kg N/ha. The treatment designated as the non-fixing control received ^{15}N labelled fertilizer at 60 kg N/ha so that the indigenous nitrogen fixing bacteria could be inhibited (Malik et al., 1988). The results of biomass yield, total nitrogen and nitrogen fixed calculated on the basis of ^{15}N isotopic dilution are summarized in Table 5. In the inoculated treatment, relatively higher biomass and nitrogen yield as compared to uninoculated treatment were observed. There was also no significant increase in rice yield in case of treatment where 60 kg N/ha was applied (data not presented).

Table 5. Effect of PGPR* inoculation on rice biomass and nitrogen fixation, calculated on the basis of ^{15}N dilution data (microplot experiment)

Treatment	Straw+grain (kg/ha)	Total N (kg/ha)	Ndfa (%)	N fixed (kg/ha)
30kg^{15}N/ha Uninoculated	15541	151	2.3	3.5
30kg^{15}N/ha Inoculated	16202	157	28.9	45.5
LSD (0.05%)	775	12	20	4

*PGPR= A mixed inoculum of *Azospirillum lipoferum* strain N-4, *Azospirillum brasilense* strain Wb-3, *Azoarcus* strain K-1, *Zoogloea* strain Ky-1 and *Pseudomonas* strain 96-51.

Such a response may be due to the available N (NH_4 11.5 mg/kg; NO_3 13.4 mg/kg) present in the microplot soil. However, statistically significant increase in the amount of nitrogen fixed was observed in the case of the inoculated treatment. The estimates were based on the calculation of "A" values of fixing and non-fixing control (treatment having 60 kg N/ha application). In case of uninoculated treatment with 30 kg N/ha application, only 3.5 kg N/ha was biologically fixed which is due to the natural rhizospheric microflora. From this data, we can infer that in case of inoculated treatment, plants are deriving nearly 30% of its nitrogen requirement from the atmospheric N, thus resulting in conservation of soil fertility by reducing the depletion of soil nitrogen. At the time of harvest, an increase in the root biomass (data not presented) in the inoculated treatments was also observed indicating the possible role of growth hormone producing bacteria included in the mixed inoculum. The survival of the inoculated bacteria was also studied by using selective media containing antibiotics, morphological characters and reaction with fluorescent antibody stain (Mehnaz et al., 1996). Maximum nitrogenase activity as estimated by acetylene reduction assays of the rice roots in the inoculated treatments was observed after one month of transplantation. Although it decreased to one third before harvesting, it was significantly higher than the uninoculated treatment (Mehnaz et al., 1996).

Conclusions

The presence of associative nitrogen fixation in grasses is a random process. There has been no evidence of the presence of any specificity of the endosymbiont for the host. However, it can be safely said that there is a group of microorganisms which colonize the root preferentially and these may thus be called "rhizotrophs". Although much work related to the inoculation of the diazotrophs has been done on the grass rhizosphere, our knowledge about root colonization is still not complete, as little is known about the mechanism of initial attachment of bacteria to the root surface. There are probably specific sites on the root surface where colonization occurs more frequently. Further studies are required to elucidate the colonization process.

References

Baldani J I, Baldani V L D, Seldin L and Döbereiner J 1986 Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root associated N_2-fixing bacteria. Int. J. Syst. Bacteriol. 36, 86–93.

Bilal R 1988 Associative nitrogen fixation in plants growing in saline environments. Ph.D. Thesis, Punjab University Lahore, Pakistan. 248 p.

Bilal R and Malik K A 1987 Isolation and identification of a N_2-fixing Zoogloea-forming bacterium from kallar grass histoplane. J. Appl. Bacteriol. 62, 289–294.

Bilal R, Rasul G, Arshad M and Malik K A 1993 Attachment, colonization and proliferation of *Azospirillum brasilense* and *Enterobacter* spp. on root surface of grasses. World J. Microbiol. Biotechnol. 9, 63–69.

Bilal R, Rasul G, Qureshi J A and Malik K A 1990 Characterization of *Azospirillum* and related diazotrophs associated with roots of plants growing in saline soils. World J. Microbiol. Biotechnol. 6, 46–52.

Boddey R M and Victoria R L 1986 Estimation of biological nitrogen fixation associated with *Brachiaria* and *Paspalum notatum* using ^{15}N labelled organic matter and fertilizer. Plant Soil 90, 265–292.

Boddey R M, Alves B J R and Urquiaga S 1996 Evaluation of biological nitrogen fixation associated with non-legumes. *In* Nitrogen Fixation with Non-Legumes. Eds. K A Malik, M S Mirza and J K Ladha, Proceedings of 7th International Symposium on BNF with non-legumes. Oct. 16–21, 1996. Faisalabad, Pakistan. *(In press).*

Boddey R M, Chalk P M, Victoria R L and Matsui E 1983 The ^{15}N isotope dilution technique applied to the estimation of biological nitrogen fixation associated with *Paspalum notatum* cv. batata in the field. Soil Biol. Biochem. 15, 25–32.

Bric J M, Bostock R M and Silverstone S E 1991 Rapid in-situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. Appl. Environ. Microbiol. 57, 535–538.

Cannon F C, Dixon R A and Postgate J R 1974 Chromosomal integration of *Klebsiella* nitrogen fixation gene in *Escherichia coli*. J. Gen. Microbiol. 80, 227–239.

Chalk P M 1991 The contribution of associative and symbiotic nitrogen fixation to the nitrogen nutrition of non-legumes. Plant Soil 132, 29–39.

Cochran W G 1950 Estimation of bacterial densities by means of the "Most Probable Number". Biometrics 6, 105–116.

Day J M, Harris D, Dart P J and Van Berkum P 1975 The Broadbalk experiment. An investigation of nitrogen gains from non-symbiotic fixation. *In* Nitrogen fixation by free-living Microorganisms. Ed. W D P Stewart Vol. 6, pp 71–84. International

Biological programme Series, Cambridge Univ. Press, Cambridge.

Döbereiner J, Marriel E and Nery M 1976 Ecological distribution of *Spirillum lipoferum* Beijerinck. Can. J. Microbiol. 22, 1464–1473.

Eskew D L, Eaglesham A R J and App A A 1981 Heterotrophic ^{15}N-fixation and distribution of newly fixed nitrogen in a rice-flooded soil system. Plant Physiol. 68, 48–52.

Fried M and Broeshart H 1975 An independent measurement of the amount of the nitrogen fixed by a legume crop. Plant Soil 43, 707–711.

Hassan U, Mirza M S, Mehnaz S, Rasul G and Malik K A 1996 Isolation and identification of diazotrophic bacteria from rice, wheat and kallar grass. *In* Nitrogen Fixation with Non-Legumes. Eds. K A Malik, M S Mirza and J K Ladha, Proceedings of 7th International Symposium on BNF with non-legumes. Oct. 16–21, 1996. Faisalabad, Pakistan. *(In press).*

Hegazi N A, Amer H A and Monib M 1979 Enumeration of N_2-fixing Spirilla. Soil Biol. Biochem. 11, 437–438.

Hurek T, Reinhold-Hurek B, van Montagu M and Kellenberger E 1991 Infection of intact roots of kallar grass and rice seedlings by "*Azoarcus*". *In* Nitrogen Fixation. Proceedings of the 5th International Symposium on Nitrogen Fixation with Non-Legumes. Eds. M Polsinelli, R Materassi and M Vincenzeni. Kluwer Academic Publishers, Dordrecht.

Kreig N R and Holt J G (Eds.) 1984 The genus *Zoogloea*. *In* Shorter Bergey's Manual of Determinative Bacteriology, 8th Edition, pp 83–84. Williams & Wilkins Co., Baltimore, USA.

Ladha J K, So R B and Watanabe I 1987 Composition of *Azospirillum* species associated with wetland rice plant grown in different soils. Plant Soil 102, 127–129.

Lethbridge G and Davidson M S 1983 Root-associated nitrogen-fixing bacteria and their role in the nitrogen nutrition of wheat estimated by ^{15}N isotope dilution. Soil Biol. Biochem. 15, 365–374.

Lifshitz R, Kloepper J W, Kozlowski M, Simonson C, Carlson J, Tipping E M and Zaleska I 1987 Growth promotion of canola (rapeseed) seedlings by a strain of *Pseudomonas putida* under gnotobiotic conditions. Can. J. Microbiol. 33, 390–395.

Malik K A, Aslam Z and Naqvi S H M 1986 Kallar grass: a plant for saline land. Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan.

Malik K A and Bilal R 1989 Survival and colonization of inoculated bacteria in kallar grass rhizosphere and quantification of N_2 -fixation. *In* Nitrogen Fixation with Non-Legumes. Eds. F A Skinner, R M Boddey and I Fendrik. pp 301–310. Kluwer Academic Publishers, Dordrecht.

Malik K A, Bilal R, Azam F and Sajjad M I 1988 Quantification of N_2-fixation and survival of inoculated diazotrophs associated with roots of Kallar grass. Plant Soil 108, 43–51.

Malik K A, Bilal R, Rasul G, Mahmood K and Sajjad M I 1991 N_2-fixation in plants growing in saline sodic soils and its relative quantification based on ^{15}N natural abundance. Plant Soil 137, 67–74.

Malik K A, Rasul G, Hassan U, Mehnaz S and Ashraf M 1993 Role of N_2-fixing and growth hormones producing bacteria in improving growth of wheat and rice. *In* Nitrogen Fixation with non-legumes. Eds. N A Hegazi, M Fayez and M Monib. pp 409–422. The Am. Univ. in Cairo Press, Cairo.

Malik K A and Zafar Y 1985 Quantification of root associated nitrogen fixation in kallar grass as estimated by ^{15}N isotope dilution. *In* Nitrogen and the Environment. Eds. K A Malik, S H M Naqvi, M I H Aleem. pp 161–171. NIAB, Faisalabad, Pakistan.

Malik K A, Zafar Y and Hussain A 1980 Nitrogenase activity in the rhizosphere of kallar grass (*Diplachne fusca* Linn Beauv). Biologia 26, 107–112.

Malik K A, Zafar Y and Hussain A 1982 Associative dinitrogen fixation in *Diplachne fusca* (kallar grass). *In* Biological Nitrogen Fixation Technology for Tropical Agriculture. Eds. P H Graham and S C Harris. pp 503–507. CIAT, Cali, Columbia.

Malik K A, Zafar Y, Bilal R and Azam F 1987 Use of ^{15}N isotope dilution for quantification of N_2-fixation associated with roots of kallar grass (*Leptochloa fusca* (L.) Biol. Fertil. Soils 4, 103–108.

Mehnaz S, Mirza M S, Hassan U and Malik K A 1996 Detection of inoculated plant growth promoting rhizobacteria in the rhizosphere of rice. *In* Nitrogen Fixation with Non-Legumes. Eds. K A Malik, M S Mirza and J K Ladha, Proceedings of 7th International Symposium on BNF with non-legumes. Oct. 16–21, 1996. Faisalabad, Pakistan *(In press).*

Qureshi J A, Zafar Y and Malik K A 1988 *Klebsiella* sp. NIAB-1: A new diazotroph, associated with the roots of kallar grass from saline sodic soils. Plant Soil 110, 219–224.

Reinhold B, Hurek T and Fendrik I 1987 Cross-reaction of predominant nitrogen-fixing bacteria with enveloped, round bodies in the root interior of kallar grass. Appl. Environ. Microbiol. 53, 889–891.

Reinhold B, Hurek T, Fendrik I, Pot B, Gillis M, Kersters K, Thielemans S and De Ley J 1987a *Azospirillum halopraeferens* sp. Nov., a nitrogen-fixing organism associated with the roots of kallar grass (*Leptochloa fusca* (L.) Kunth). Int. J. Syst. Bacteriol. 37, 43–51.

Reinhold B, Hurek T, Niemann E G and Fendrik I 1986 Close association of *Azospirillum* and diazotrophic rods with different root zones of kallar grass. Appl. Environ. Microbiol. 52, 520–526.

Reinhold-Hurek B, Hurek T, Gillis M, Hoste B, Vancanneyt M, Kersters K and De Ley J 1993 *Azoarcus* gen. nov., nitrogen fixing proteobacteria associated with roots of kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov., and *Azoarcus communis* sp. nov. Int. J. Syst. Bacteriol. 43, 574–584.

Rennie R J 1980 Isotope dilution as a measure of dinitrogen fixation by *Azospirillum brasilense* associated with maize. Can. J. Bot. 58, 21–24.

Rennie R J 1981 A single medium for the isolation of acetylene reducing (dinitrogen fixing) bacteria from soil. Can. J. Microbiol. 27, 8–14.

Rennie R J and Larson R I 1979 Dinitrogen fixation with disomic chromosome substitution lines of spring wheat. Can. J. Bot. 57, 2771–2775.

Rennie R J, de Freitas J R, Ruschel A P and Vose P B 1983 ^{15}N dilution to quantify dinitrogen (N_2)-fixation associated with Canadian and Brazilian wheat. Can. J. Bot. 61, 1667–1671.

Roger P A and Ladha J K 1992 Biological N_2 fixation in wetland rice fields: estimation and contribution to nitrogen balance. Plant Soil 141, 41–55.

Ruschel A, Henis Y and Salati E 1975 Nitrogen ^{15}N tracing of N_2-fixation with soil grown sugarcane seedlings. Soil Biol. Biochem. 5, 83–89.

Sandhu G R and Malik K A 1975 Plant succession – A key to utilization of saline soils. Nucleus 12, 35–38.

Shrestha R K and Ladha J K 1996 Genotypic variation in promotion of rice dinitrogen fixation as determined by nitrogen-15 dilution. Soil Sci. Soc. Am. J. 60, 1815–1821.

Sundaram S, Arunkumari A and Klucas R V 1988 Characterization of azospirilla isolated from seeds and roots of turf grass. Can. J. Microbiol. 34, 212–217.

Tarrand J J, Krieg N R and Döbereiner J 1978 A taxonomic study of *Spirillum lipoferum* group with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Can. J. Microbiol. 24, 967–980.

Tien T M, Gaskins M H and Hubbell D H 1979 Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.) Appl. Environ. Microbiol. 37, 1016–1024.

Van Berkum P 1980 Evaluation of acetylene reduction by excised roots for the determination of nitrogen fixation in grasses. Soil Biol. Biochem. 12, 141–145.

Ventura W and Watanabe I 1983 ^{15}N dilution of accessing the contribution of nitrogen fixation to rice plant. Soil Sci. Plant Nutr. 29, 123–131.

Witty J F 1979 Acetylene reduction assay can overestimate nitrogen fixation in soil. Soil Biol. Biochem. 11, 209–210.

Zafar Y, Ashraf M and Malik K A 1986 Nitrogen fixation associated with the roots of kallar grass (*Leptochloa fusca* (L.) Kunth). Plant Soil 90, 93–106.

Zafar Y, Malik K A and Niemann E G 1987 Studies on N_2-fixing bacteria associated with salt tolerant grass *Leptochloa fusca* (L.) Kunth. Mircen J. Appl. Microbiol. 3, 45–56.

Zimmer W, Roeben K and Boothe H (1988) An alternative explanation for plant growth promotion by bacteria of the genus *Azospirillum*. Planta 176, 333–342.

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